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Title: **Three-dimensional antennal lobe atlas of the Oriental fruit moth, *Cydia molesta* (Busck) (Lepidoptera: Tortricidae): comparison of male and female glomerular organization**

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Abstract

The Oriental fruit moth *Cydia molesta* is an important pest insect and the behavioural role of olfactory signals such as pheromones and plant volatiles have been studied extensively for both sexes. To further understand odour processing, however, a detailed knowledge of the anatomy of the olfactory system is crucial. In the present study, an atlas of the antennal lobe (AL) is presented based on the three-dimensional reconstructions of both ALs of three male and three female brains using neuroanatomical and computational approaches. We identified 48 to 49 "ordinary" glomeruli and one large glomerulus situated at the entrance of the antennal nerve in males, and 49 to 52 "ordinary" glomeruli and one large glomerulus in the ventro-medial part of the AL in females. Anomalous supernumerary, anomalous missing and sexually dimorphic glomeruli were found in the studied individuals in greater number than in other lepidopteran species. Male and female maps were compared as to glomerular size and position and forty-five glomeruli could be matched, indicating a conserved glomerular pattern between the sexes. Three additional glomeruli were sexually dimorphic in size and five male and six female specific glomeruli were also found. Palp backfills resulted in the staining of a unique glomerulus in both sexes identified as the sexually dimorphic glomerulus 45. This glomerulus was never stained from antennal backfills, which stained the other glomeruli of the AL. The three-dimensional atlas will now be used to elucidate the functional role of individual glomeruli in both sexes of *C. molesta*.

Abbreviations

3D – Three-dimensional

AL – Antennal lobe

AN – Antennal nerve

Cu – Cumulus

MGC – Macroglomerular complex

NGS – Normal goat serum

ORN – Olfactory receptor neuron

PBS – Phosphate buffer solution

PN – Projection neuron

*Z*8-12:Ac – (*Z*)-8 dodecenyl acetate

*E*8-12:Ac – (*E*)-8 dodecenyl acetate

*Z*8-12:OH – (*Z*)-8 dodecen-1-ol

12:OH – dodecan-1-ol

Introduction

In insects, odour information is detected and coded in olfactory receptor neurons (ORNs) housed in sensilla mainly located on the antenna. ORNs send axonal projections to the first structure that processes olfactory information in the brain, the antennal lobe (AL). The ALs are divided into structural units named glomeruli. These glomeruli emerge as important functional structures, in which synaptic interactions take place, and which are accessible for experiments due to their small number and large size. The distinctness of glomeruli varies greatly among different species and their number is species-specific (e.g. Rospars, 1988; Hildebrand and Shepherd, 1997; Rospars and Hildebrand, 2000). For most insect species, the AL contains between 40 and 100

glomeruli, as opposed to the vertebrate primary olfactory centre, the olfactory bulb, that often contains more than one thousand glomeruli. Nonetheless important similarities have been found in the neuroanatomy and physiology between the olfactory systems of mammals and insects, (Hildebrand and Shepherd, 1997), and an increasing number of studies suggests that the quality of an odour stimulus is represented across the glomerular array, both in insects (Joerges *et al.*, 1997; Vickers *et al.*, 1998; Mustaparta, 2005) and in vertebrates (Jourdan *et al*., 1980; Kauer, 2002; Bozza *et al*., 2002).

In Lepidoptera, sex pheromone-related information is mostly processed within the macroglomerular complex (MGC), a group of glomeruli that are male-specific and typically enlarged in this sex, whereas plant odour-related information is known to be processed in the so-called "ordinary" glomeruli present in both males and females (Anton and Hansson, 1994, 1995; Christensen and Hildebrand, 2002). The number of glomeruli forming the MGC is often similar to the number of behaviourally relevant pheromone components, with each ORN type projecting to one MGC glomerulus in several moth species (*e.g.* Hansson *et al.*, 1992; Ochieng *et al.*, 1995; Todd *et al.*, 1995; Berg *et al.*, 1998). By contrast, the representation of non-pheromone odours is less well understood. Although some attempts have been made to stain plant-odour responding ORNs from the antennae of different moth species (Hillier et al., 2006; Hillier and Vickers, 2007; Todd and Baker, 1996) without succeeding in a clear functional mapping, the specificity of non-pheromone receptor projections has been primarily examined indirectly via olfactory membrane receptor expression (*e.g*. Gao *et al.*, 2000; Vosshall *et al.*, 2000; Bhalerao *et al.*, 2003) and optical imaging studies in moths, fruit flies and honeybees (Galizia *et al.*, 2000; Carlsson *et al.*, 2002; Meijerink *et al.*, 2003; Wang *et al.*, 2003). In these studies, functional representation of general odours has been shown to be similar in both sexes (Carlsson *et al*., 2002). Anatomical matching of isomorphic glomeruli between the sexes has been carried out in the cockroach *Blaberus craniifer* (Rospars and Chambille, 1981), the noctuid moth *Mamestra brassicae*

(Rospars, 1983), the sphingid moth *Manduca sexta* (Rospars and Hildebrand, 2000), and in heliothine moths (Skiri *et al*., 2005).

To better understand the spatial component of odour processing in glomeruli, it is necessary to know their exact arrangement within the AL. For this purpose, three-dimensional (3D) maps have been constructed for several insect species (Rospars, 1983; Flanagan and Mercer, 1989; Rospars and Chambille, 1989; Stocker *et al*., 1990; Galizia *et al.*, 1999; Laissue *et al.*, 1999; Rospars and Hildebrand, 2000; Chiang *et al.*, 2001; Berg *et al.*, 2002; Sadek *et al*., 2002; Smid *et al.*, 2003; Greiner *et al.*, 2004; Ignell *et al*., 2005; Huetteroth and Schachtner, 2005; Masante-Roca *et al.* 2005; Skiri *et al.*, 2005; Ghaninia *et al*., 2007; Zube *et al*., 2008).

The Oriental fruit moth *Cydia molesta* (Busck) (Lepidoptera: Tortricidae), (previously *Grapholita molesta*), is a major pest of stone fruits worldwide and recently also of apples and pears (Rothschild and Vickers, 1991; Il'ichev *et al.*, 2004; Reis *et al.*, 1988; Hickel and Ducroquet, 1998; Kovanci *et al.*, 2004). Its sex pheromone blend comprises four components, (*Z*)-8 dodecenyl acetate (*Z*8-12:Ac), (*E*)-8 dodecenyl acetate (*E*8-12:Ac), (*Z*)-8 dodecen-1-ol (*Z*8-12:OH) and dodecan-1-ol (12:OH) (Roelofs *et al.*, 1969, Cardé *et al.*, 1979), the first three being required to evoke pheromone-mediated flight in males (Baker *et al*., 1981; Charlton and Cardé, 1981; Linn and Roelofs, 1983). Pheromone-based techniques are well established to monitor *C. molesta*, and since the implementation of mating disruption, the effect of the sex pheromone on the behaviour of males has been extensively studied (Charlton and Cardé, 1981; Figueredo and Baker, 1991; Rumbo and Vickers, 1997; Stelinski *et al.*, 2005). Also the effect of male pre-exposure to the sex pheromone has been studied. Stelinski *et al.* (2005) found that mean durations of sustained flights of pre-exposed males were shorter than for naïve males and that the effect of the pheromone pre-exposure decayed over time. The effect of sex pheromone on the behaviour of females has only recently begun to be investigated: only the major component (*Z*8-12:Ac) has been reported to have an effect in females by advancing their calling

time by two hours (Stelinski *et al.*, 2006). Plant derived stimuli are no less important for phytophagous insects than is the sex pheromone. Mated females were shown to fly to peach and apple fruits and to butyl hexanoate, a major apple volatile in a double choice arena test (Natale *et al*, 2004; Piñero and Dorn, 2007).

Although the behaviour of *C. molesta* has been well studied, the structure and function of its peripheral and central olfactory system are largely unknown. Nonetheless, calcium imaging studies showed that benzaldehyde - a minor constituent of a host plant-derived synthetic mixture - that elicits a behavioural response in mated females, plays a key role in the behavioural discrimination and in the neural representation of mixtures (Piñero *et al*., 2007). The behavioural data available makes this species well suited for functional studies of plant and pheromone processing and its plasticity.

In the present study we established the 3D glomerular map for males and females of *C. molesta* as a tool for future functional studies. We aimed at a precise comparison of male and female maps in a species where detection of pheromone and plant compounds is well described in both sexes. Backfills from palps and antennae were performed to differentiate between target glomeruli of functionally discrete receptor neuron populations. Precise maps are a prerequisite to understand the discrimination of olfactory signals. The 3D map will serve to identify target glomeruli of physiologically characterized and stained ORNs and projection neurons (PNs) in this species. It will also provide a common frame to interpret calcium-dependent responses in optical imaging studies and to localize glomeruli that are involved in different kinds of plasticity.

Materials and Methods

Insects

Pupae of *Cydia Molesta* were obtained from Piacenza, Italy (courtesy of Dr. Fabio Molinari). A rearing on an artificial diet was maintained at Lleida, Spain, and the adults were allowed to emerge in an environmental chamber kept under a 16L:8D cycle and 25±1°C temperature. Male and female adults used for anatomical experiments were no more than three days old.

Antibody staining procedure

In order to visualize individual glomeruli and study the anatomy of *C. molesta* we first punctured the insects head capsule and then fixed it for three hours at room temperature in a solution of 4% paraformaldehyde in 0.1M phosphate buffer solution (PBS) at pH 7.4 to facilitate the dissection. Once dissected, the brains were kept overnight in a fixation solution of 4% paraformaldehyde with 0.25% TritonX-100 in PBS (PBST) at 4°C. After fixation the brains were rinsed in PBST and left in 2% normal goat serum (NGS; Sigma-Aldrich Quimica S.A., Madrid, Spain) in PBST for 2 hours. Afterwards they were incubated for 48h on a rotator in a synapsin antibody produced in mouse (DSHB, IA, USA, Klagges *et al.*, 1996) diluted 1:50 in PBST with 2% NGS at 4°C. After primary incubation the brains were rinsed in PBST and incubated in Alexa fluor 488 goat anti-mouse conjugate (Invitrogen™) applied at 1:150 in PBST and kept for 48h on a rotator at 4°C. The brains were then rinsed with PBST and cleared in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) for 24h and mounted as wholemounts on glass slides with a spacer (0.12 mm) between the slide and the cover slip. They were visualized under a laser scanning confocal microscope (see below).

Palp and antennal backfills

One and two days old male and female moths were restrained in a plastic pipette tip with the head protruding. The head was immobilized with dental wax. The pipette tip was then placed horizontally inside a Petri dish containing a moist paper tissue. The tip of the antennae or the tip of the palp was cut and a glass capillary filled with 1% solution of neurobiotin (Neurobiotin™ Tracer, Vector Laboratories, Burlingame, CA, USA) in 0.25 M KCl was also placed horizontally allowing the cut end of the insect antennae or the palp to enter the glass capillary. The Petri dish was then placed at 4ºC and left for 24h for antennal backfills and 6h for palp backfills.

Afterwards neurobiotin-stained brains were dissected in Millonig's buffer (pH 7.2) and fixed in 4% paraformaldehyde in Millonig's buffer at room temperature overnight. The brains were then rinsed in Millonig's and dehydrated and rehydrated in ethanol and propylene oxide to increase membrane permeability. Neurobiotin was then visualized through incubation in buffered Oregon green-avidin conjugate (Oregon Green®, Invitrogen™, Barcelona, Spain) with 0.25% TritonX and 1% bovine serum albumin. After incubation, the brains were again rinsed in Millonig's and cleared and mounted in Vectashield as described above.

Confocal microscopy

Wholemount brains were viewed with a FluoView 500 Olympus confocal microscope (Hamburg, Germany) equipped with an argon laser that permitted the visualization of structures labelled with Alexa fluor 488 (Fig 1). For the overview scanning of the whole brain a 20x dry objective was used with optical sections at 2 µm whereas the detailed scanning was done with a 40x dry objective at 1µm optical sections. All confocal images were scanned and stored at a resolution of 1024 x 1024 pixels.

Maximum projections were done from partial stacks of backfill image series obtained with the confocal microscope with Image J software (NIH, USA).

Segmentation and measurements on sections

The brains that presented good fluorescent staining in all sections of the entire stack were selected for three-dimensional reconstruction. In both the right and the left ALs of three different males (*M*1, *M*2 and *M*3) and three females (*F*1, *F*2 and *F*3) the outlines of each individual glomerulus were manually delineated in every optical section of the high magnification scans using a custom-made program (Kiêu K, Couton L and Rospars JP) developed in the Matlab language (The MathWorks, Natick, MA, USA). The volume v and the coordinates x, y, z of the centre of mass of each glomerulus were calculated from its outlines. The data relative to a glomerulus were then reduced to four values: the coordinates x, y, z and the radius R of the sphere having the same volume. The x and y axes corresponded to the bottom and left edges of each image, respectively. The z axis corresponded to the succession of the intersections of x and y axes, at the bottom-left, and defined the location of every section within the stack. The z-dimension (thickness) had to be corrected by a factor of 1.6 because of the refractive index mismatch caused by air objectives (Bucher *et al*. 2000). The voxel size obtained for the overview scanning was of 0.550 x 0.550 x 3.2 µm (x, y, z). For the detailed scanning the voxel size was of 0.275 x 0.275 x 1.6 µm (x, y, z).

Identification and final coordinates of glomeruli

To identify glomeruli and calculate their standardized coordinates we proceeded as explained previously (Rospars, 1983; Couton et al, submitted). Only a summary will be given here. After all visible glomeruli had been delineated in both ALs, a preliminary number was given to each of them. However, two large glomeruli, one at the entrance of the antennal nerve (AN) in males and another one located medio-ventrally in females could be recognized immediately in all

individuals of the same sex. These glomeruli were given the same number in all three individuals of the same sex, and were used as landmark glomeruli.

Then a comparison was made to match homologous right and left glomeruli (intraindividual). This comparison was achieved by comparing 1) the confocal sections directly at different levels of the z axis throughout the AL, 2) realistic 3D reconstructions and 3) standardized views showing the ALs from different sides (lateral, medial, anterior, posterior, dorsal and ventral) after correction of the orientation of the brains with respect to their plane of symmetry. In these views the left ALs are represented as their mirror image with respect to the plane of symmetry of the brain to be directly comparable to the right ALs. After matching right and left glomeruli in ALs of the same individual an average AL was produced by calculating the mean coordinates and mean radii of the matched glomeruli.

The interindividual matchings were achieved in three steps. First, we compared the standardized view of the 3 average ALs of males. To compensate for the different optical sectioning planes in different preparations we rotated the average ALs around the medio-lateral X axis which is perpendicular to the plane of symmetry. At this stage the position of each glomerulus is defined in a coordinate system with its origin at the AL centre and with axes X medio-lateral, Y postero-anterior and Z ventro-dorsal. We calculated an average male AL based on the means of coordinates X, Y, Z and radii of all matched glomeruli. Secondly we did the same for the females and oriented the ALs identically to the male ALs in order to obtain an average female AL. Finally we compared the standardized views of the two reconstructed average ALs (male and female) to determine eventual differences between them. We additionally checked these intersexual matchings on the 6 average male and female ALs.

Statistical tests

The sizes of matched male (6 radii) and female (6 radii) glomeruli were compared by the nonparametric Wilcoxon test at the 1% significance level. The glomeruli for which this test showed a significant difference were considered as sexually dimorphic. The global equality in size of homologous glomeruli in two lobes was tested at the 5% significance level using the coefficients of correlation of their radii. The conservation of their spatial location was tested in the same way using the distance of each glomerulus to the centre of the lobe in which it resides. All tests were done with the Matlab Statistical Toolbox.

Nomenclature

For naming glomeruli the following rules were used: glomeruli found in several lobes were given a number (1 to 45), those found in a single sex (sex specific) were given a letter ("a" to "e" in males, "f" to "n" in females). Finally, the anomalous glomeruli found in a single lobe were also given a letter ("o" to "q"). So all numbered glomeruli were found in both sexes, whereas glomeruli named with a letter were found in only one sex, one individual or one lobe.

Results

Three-dimensional reconstruction

The small size of the *C. molesta* brains made it possible to easily visualize the entire AL as a single stack of sequential optical sections (Figs. 1, 2). The ALs had a more or less spherical shape and their antero-posterior diameter was of 70 to 85 μm for males and of 60 to 75 μm for females. Left and right ALs were situated touching each other and the main cell body cluster was located ventrally in both sexes (Figs. 1, 2). The ordinary glomeruli surrounded a central fibre

core devoid of any glomeruli (Figs. 1, 2). Most glomeruli were uniform in shape, size and relative position when comparing different ALs and could therefore be identified and recognized individually.

Male antennal lobes

In the six male ALs studied we found 49 to 50 glomeruli. Forty seven of them could be systematically identified in all six ALs, one of them being the enlarged glomerulus located at the antennal nerve entrance (44 = Cumulus, Cu) (Fig. 1, 3a). The remaining glomeruli are anomalous considering the studied samples, and belong to two categories, missing and supernumerary (Table 1, left columns). We considered a glomerulus as missing when it was absent from a single individual or from a single AL. Glomerulus 42 is missing in both lobes of *M*2 and is the smallest glomerulus in *M*1 and *M*3 with a radius R = 9 µm. Glomerulus 36 is missing in the left lobe of *M*3, and glomerulus 40 in its right lobe (Table 1). Conversely, we considered a glomerulus as supernumerary when it was present in a single individual, like "q" which was found only in *M*2 (Table 1) and it is also a small glomerulus (R = 9 µm).

The radii of the male glomeruli are mostly in the range of 9 to 16 µm (Fig. 4, thick line). The Cu was the biggest structure within all ALs with a radius of 25, 22 and 27 μm in *M*1, *M*2 and *M*3, respectively. It is bordered by two large glomeruli, one laterally, close to Cu (11 with R = 16 µm) and another dorsally (12 with R = 15 µm) (Fig. 3c, e). Two of the smallest male glomeruli are on opposite sides of the most posterior AL region, one ventral (35 with R = 9 µm) and the other dorsal (42 with R = 10 µm) (Fig. 3b, d).

The sizes of homologous glomeruli in left and right ALs of the same brain and across male individuals were very similar, as shown by their coefficients of correlation (Table 2, Fig. 5a, c). The same conclusion holds for the distances of homologous glomeruli to the centre of their

respective AL (Table 2, Fig 5b, d), meaning that identified glomeruli from the same and different individuals had similar sizes and were situated in a similar position.

Female antennal lobes

In the six female ALs studied we found 50 to 53 glomeruli. Forty-seven of them were identified in all female ALs. Additional glomeruli were found only in some ALs within our sample (Table 1, right columns). Like in males, these anomalous glomeruli are supernumerary or missing. Two are apparently supernumerary: “o” (present only in the right lobe of *F*2), and “p” (only in the right lobe of *F*3) (Table 1). Other glomeruli are missing unilaterally or bilaterally in one individual: glomerulus “m” is missing in *F1*, “l” and 41 are missing in *F*2, and “n” and 37 in *F*3 (Table 1). Interestingly, “l” is the smallest female glomerulus (R = 7 µm) (Fig. 3g-i).

The best identifiable structure was an enlarged glomerulus located medio-ventrally, the large glomerulus 45 (Figs. 2, 3i, j). This exceptional glomerulus had a radius of 21, 20 and 23 μm in females *F*1, *F*2 and *F*3, respectively. The smallest female glomeruli are ”l”, “n” (R = 9 µm) and 14 (R = 10 µm). Like in males, the radii of the other female glomeruli are in the range 9 to 16 µm (Fig. 4, thin line) and the intra- and inter-individual correlations of the radii and of the distances to the AL centre of homologous glomeruli are highly significant (Table 2).

Comparison of male and female antennal lobes

The systematic comparisons of male and female ALs led to distinguish three main qualitative classes of glomeruli within our sample: sex isomorphic, sex dimorphic and sex specific.

The isomorphic glomeruli are those present in both sexes in the same position and with the same size. They form the largest class (35 glomeruli). In both sexes glomeruli 1, 2 and 4 are

situated close to each other in all ALs studied (Fig. 3c, i). Two of the largest and most elongated glomeruli (11 and 12) are situated antero-ventrally and dorsally. The most anterior glomeruli seem more densely packed than glomeruli in the rest of the AL. Seven other glomeruli (36 to 42) can be put in this class although they are anomalous missing in one or two individuals. In particular, this is the case of glomeruli 38 and 39 of female *F*3, which were not found in *F*1 and *F*2. In fact the male-female comparisons suggest that they correspond to male glomeruli 38 and 39. For this reason, they were considered as anomalous missing in two of the females (*F*1 and *F*2) and present in the other four individuals (*M*1-3 and *F*3). The radius histograms of isomorphic glomeruli are similar in males and females with an approximately Gaussian distribution in the range 9 to 16 µm (Fig. 4).

Sex dimorphic glomeruli are also present in both sexes but with significantly different radii (*p*-value less than 1%). We found 3 glomeruli in this class (Table 1), which are at least 25% larger in one sex than in the other. One of them, 44 (= Cu) (Fig. 3a, g), is 93% larger in males than in females, with average radii of 25 and 13 µm respectively. It is located at the entrance of the AN in both sexes. Two glomeruli, 43 and 45, are larger in females than in males. The average radii of glomerulus 43 are 13 µm in males and 16 µm in females, i.e. 25% larger in females. Glomerulus 45, located medio-ventrally (Fig. 3c, i), is 37% larger in females (16 µm in males and 22 µm in females). Two of the sexually dimorphic glomeruli (44 and 45) are far away from the Gaussian distribution of the other glomeruli (Fig. 4), whereas the third one, glomerulus 43, is included in the distribution, indicating that sexual dimorphism is not restricted to "macroglomeruli".

Sex-specific glomeruli were found in only one sex, five in males ("a" to "e") and six in females ("f" to "k"). Three "anomalous" glomeruli ("l", "m" and "n") can be put in the female-specific class, when considering our sample; each was missing bilaterally in one female and in all males. These glomeruli were also found in different locations within the AL.

With this interpretation, which results from the study of 12 ALs, the 10 anomalous missing glomeruli listed in Table 1 can be finally assigned to two of the "regular" classes, the sex isomorphic (36 - 42 missing in one or two individuals), and the female specific ("l", "m", "n" missing in one female).

Finally, the location and size of the 45 homologous (isomorphic and sexually dimorphic) glomeruli were globally compared in males and females, using regression plots (Fig. 5e, f) and coefficients of correlation (Table 2). Both methods show that the location of glomeruli – as measured by their distance to the centre of the lobe – are well conserved between sexes, although less well than between individuals of the same sex. Similarly, the intersexual coefficient of correlation of the radii of homologous glomeruli in the male and the female average ALs were found larger than the intrasexual coefficients of correlation. However, all correlations were still significant (Table 2, Fig. 5e, f).

Palp and antennal backfills

Out of thirty-nine attempted backfills done from the palp, nineteen (49%) were successful, and out of twelve attempted backfills from the antennae, seven (58%) resulted in stained fibres in the brain. Unilateral palp backfills revealed bilateral projections ascending to the AL, in both males and females. Within each AL, only one glomerulus in a medio-ventral position received stained fibres (Figs. 6A-B). In males, the glomerulus stained was identified as number 45, which, according to the male-female comparison, also corresponds to glomerulus 45, stained in our palp backfill preparations in females (Figs. 6a, b).

Unilateral antennal backfills revealed ipsilateral staining in a large number, but not all glomeruli in both males and females. In none of the successful antennal backfills, we found stained branches in glomerulus 45 in males or in females (Figs. 6c, d).

Discussion

In the present paper, a complete AL atlas for both sexes of the tortricid moth *C. molesta* is presented based on the systematic anatomical matching of glomeruli within and between the sexes. Although sexual isomorphism of so-called "ordinary" glomeruli has been known for a long time (Rospars and Chambille, 1989), we show here that, even though a large percentage of glomeruli seem to be isomorphic between sexes, there are still many differences found as to the presence, absence or size of glomeruli for this lepidopteran species, permitting us for our sample to distinguish anomalous supernumerary, anomalous missing, sexually dimorphic, and sexually-specific glomeruli.

Number of glomeruli in Cydia molesta

The AL structure of *C. molesta* resembles the glomerular organization of other insects, i.e., glomeruli that surround a central fibre core. It is known that the number of glomeruli varies among species ranging from ~50 to 61 in mosquitoes (Ignell *et al.*, 2005; Ghaninia *et al.*, 2007), 50 in fruit flies (Laissue *et al.*, 1999; Couto et al., 2005; Fishilevich & Vosshall, 2005), 99 in cockroaches (Chiang *et al.*, 2001), 156 in honeybees (Flanagan and Mercer, 1989; Galizia *et al.*, 1999), and up to 1000 in locusts or social wasps (see references in Ignell *et al.*, 2001). In Lepidoptera the number of glomeruli is rather constant, independently of the family or the size of the animals, and varies between 60 and 70 (Rospars, 1983; Rospars and Chambille, 1989; Rospars and Hildebrand, 1992, 2000; Berg *et al.*, 2002; Sadek *et al.*, 2002; Ai and Kanzaki, 2004; Greiner *et al.*, 2004; Masante-Roca *et al.*, 2005).

In *C. molesta* the number of "ordinary" glomeruli found, 50 in males and 54 in females (not including anomalous supernumerary glomeruli) is almost the same as that found in the sibling species *Cydia pomonella*, 50 in males and 52 in females (Ansebo, 2004). It is slightly lower than the numbers found in other lepidopteran species: 64-68 in various Noctuid species (Rospars, 1983; Berg *et al*., 2002; Sadek *et al*., 2002; Greiner *et al*., 2004), 64 in the Sphingid *Manduca sexta* (Rospars and Hildebrand, 1992, 2000) and 60-71 in the Tortricid moth *Lobesia botrana* (Masante-Roca *et al*., 2005). The mentioned lepidopteran species investigated so far belong to distantly related families, and differences in the number of glomeruli within the AL are therefore not surprising. However, the two Tortricid genera *Cydia* and *Lobesia* are closely related and the difference in the number of glomeruli was unexpected. An explanation of this difference might be different life styles of the investigated species. *C. molesta* and *C. pomonella*, which are oligophagous insects, considered as major pests of peach and apple, respectively, may use a narrower range of olfactory cues to find their hosts than *Lobesia botrana*, a polyphagous insect which can develop on more than forty species of plants (Gabel *et al.*, 1992; Ben-Yehuda *et al.*, 1993; Katerinopoulos *et al.*, 2005) even though it is considered as a major grapevine pest. In an insect species, the number of glomeruli is closely related to the number of expressed olfactory receptors (Couto et al., 2005), and consequently to the number and complexity of odours they can discriminate. This is confirmed by data from highly social insects like bees and ants, which have relatively large numbers of glomeruli and use a large variety of chemical cues (Galizia *et al.*, 1999; Kleineidam *et al.*, 2005).

Sexual dimorphism in the AL of Cydia molesta

We found that a high percentage of glomeruli (88% in males – 45 out of 50 – and 83% in females – 45 out of 53) could be matched in the same position in both male and female ALs.

This population can be subdivided in two classes, the sex-isomorphic glomeruli (42) and the sex-dimorphic ones (3). Moreover, there are, in both sexes, glomeruli present that cannot be matched across sexes which form a class of sex-specific glomeruli (5 in males and 9 in females for our sample).

Macroglomeruli in males. In species, which use sex pheromone communication, such as moths, large glomeruli are commonly found. These glomeruli have been shown to receive exclusive input from ORNs sensitive to sex pheromone and the number of macroglomerular substructures in Lepidoptera is thought to be correlated with the number of behaviourally active components forming the pheromone blend of the respective species (Hansson *et al.*, 1992; Ochieng *et al.*, 1995; Todd *et al.*, 1995; Berg *et al.*, 1998). In *C. molesta* we found one enlarged glomerulus at the entrance of the antennal nerve in males. In *C. molesta* males, at least three pheromone components, Z8-12:Ac, E8-12:Ac and Z8-12:OH, are needed to elicit upwind flight (Figueredo and Baker, 1991; Lacey and Sanders, 1992; Rumbo and Vickers, 1997; Stelinski *et al.*, 2005). Therefore we hypothesize that other glomeruli may be part of a more complex macroglomerular structure, which cannot be identified by anatomical criteria alone.

Female enlarged glomeruli. Even though plant volatiles generally seem to be more important to females than the sex pheromone, pheromone auto-detection is known from a few species (e.g. Ljungberg *et al.,* 1993). In the case of *C. molesta*, females advance their calling period by two hours when they detect the sex pheromone released by conspecific females (Stelinski *et al.*, 2006). No functional study, in males or females, has yet been done to know which glomeruli are involved in the processing of the sex pheromone or plant volatiles in this species. Although antennal electrophysiological responses and a behavioural activation by the major pheromone component (Z8-12:Ac) have been demonstrated (Stelinski *et al.*, 2006), there is no

macroglomerulus-like structure located at the entrance of the antennal nerve in females. However, there is a glomerulus (44, Fig. 3) located in the same position as the male cumulus at the entrance of the AL. In the noctuid moth *Spodoptera littoralis*, females have receptor neurons, which detect their own pheromone as well as behavioural antagonists (Ljunberg *et al.*, 1993). Such ORNs sensitive to the major sex pheromone component of their own pheromone were shown to project to a small glomerulus located at the entrance of the AL (Ochieng *et al.*, 1995). Moreover, intracellular recordings showed that projection neurons arborizing in the female glomeruli that occupy a position equivalent to the male MGC always responded to the pheromone components (Anton and Hansson, 1994). We can speculate that the same situation occurs in *C. molesta* as in *S. littoralis*. However, the functional role of glomeruli in both males and females can only be elucidated by electrophysiological studies with subsequent staining or imaging experiments.

Other glomeruli differing between the sexes. Male moths need to find mating partners and they therefore have specific, often enlarged, glomeruli dedicated to sex pheromone processing. Mated females, on the other hand need to find oviposition sites. Therefore we suppose that plant volatiles are more important for females than the sex pheromone and this could account for the slightly higher number of "ordinary" glomeruli found in females than in males and also for the two sexually dimorphic glomeruli (43 and 45) which are larger in females than in males.

One of these glomeruli (45) has a larger size than "ordinary" glomeruli and is unlikely to be a part of the pheromone sensitive glomeruli due to its position. According to our palp and antennal backfill preparations, this glomerulus, both in males and in females, receives input from the palp and not from the antennae. It is found in a position corresponding to the "labial pit organ glomerulus" previously described for *M. sexta* (Kent *et al*., 1986; Rospars and Hildebrand, 2000) or *H. assulta* and *H. virescens* where it was easily identified for its large size and ventral position

(Berg *et al.*, 2002). Receptor neurons from the maxillary palps and labial palps of holometabolous insects usually target specific ventral glomeruli within the AL and have been shown to process information on CO_2 levels in the environment (Guerenstein and Hildebrand, 2007). More specifically, receptor neuron projections from the labial pit organ in moths and from maxillary sensilla in flies arborise uni- or bilaterally within one to three medio-ventrally situated glomeruli in each AL (Anton and Homberg, 1999; Guerenstein *et al*., 2004; Ignell *et al*., 2005; Ghaninia *et al*., 2007; Guerenstein and Hildebrand, 2007).

The coefficients of correlation of glomerular radii in intra-sexual comparisons are larger than the coefficient in the inter-sexual comparison (Table 2). This indicates that the so-called 'isomorphic' glomeruli are not identical in both sexes, although the pairwise differences in size between homologous male and female glomeruli are not statistically significant at the 1% level when compared one at a time. The inter-sexual differences appear clearly only when studied collectively with plots (compare Figs. 5c and 5e) and coefficients of correlation (Table 2). This diffuse kind of sexual dimorphism was previously observed in other species as well (Rospars and Chambille, 1989). It may reflect sexual differences in antennal olfactory sensilla, so that ORNs, expressing a given receptor type, which project in any given glomerulus, might differ more in number between sexes than within the same sex.

Conclusions

In the case of *C. molesta* data are available on the effect of pheromone and specific host-plant compounds on the behaviour of males and females, providing an excellent basis for functional studies. The map of the AL of *C. molesta* will be a useful tool for the identification of target glomeruli of physiologically characterized ORNs and/or AL neurons and will serve as a tool for

future functional studies allowing a better interpretation of the individual steps of the olfactory code.

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Figure Legends

Fig. 1 Series of confocal sections from anterior (**a**) to posterior (**f**) at different depths (z= distance from anterior pole of the AL) through the left antennal lobe of *Cydia molesta M*1, reconstructed in Fig. 3a-f. AN, antennal nerve; CB, cell body cluster; d, dorsal; l, lateral; m, medial; v, ventral; 1 to 45 and a to e, male glomeruli (see Table 1). Scale bar = 50 µm.

Fig. 2 Series of confocal sections from anterior (**a**) to posterior (**e**) at different depths (z= distance from anterior side of the AL) through the left antennal lobe of *Cydia molesta F*1, reconstructed in Fig. 3g-l. AN, antennal nerve; CB, cell body cluster; d, dorsal; l, lateral; m, medial; v, ventral; 1 to 45 and f to n, female glomeruli (see Table 1). Scale bar = 50 µm.

Fig. 3 Three-dimensional map of the left antennal lobe of male *M*1 (**a-f**) and female *F*1 (**g-l**). Graphs show the views perpendicular to the medio-lateral X, postero-anterior Y and ventro-dorsal Z axes. Small sketches within each view show how the ALs (red circles) are oriented with respect to the brain. Glomeruli coloured according to the categories in table 1. Yellow: anomalous missing (not present in at least one individual of the two sexes: 36-42, l, n). Red: male specific (a-e). Blue: female specific (f-k). Green: sexually dimorphic (43-45). Grey: sexually isomorphic (1-35). Anomalous supernumerary (present in only one individual) not shown. AN, antennal nerve; CB, cell body cluster; OL, optic lobe; SOG, suboesophageal ganglion; isomorphic glomeruli, same as in Fig. 2. Scale bar = 50 µm.

Fig. 4 Histograms of glomerular sizes in males (thick line) and females (thin line). In both sexes the radius of each identified glomerulus was calculated as the mean of the available measurements (6 per sex for non-anomalous glomeruli). Almost all glomeruli follow a normal

distribution in the range 9 to 16 µm. The two rightmost glomeruli are the sexually dimorphic 44 (25 µm in males) and 45 (22 µm in females).

Fig. 5 Comparison of sizes and spatial locations of homologous glomeruli in *Cydia molesta.* Location is quantified by the distance of each glomerulus to the centre of the corresponding AL. Radii (**a**) and distances (**b**) between the right and left antennal lobes of *M*1 (intra-individual comparison). Radii (**c**) and distances (**d**) between averages of right and left lobes for males *M*1 and *M*2 (inter-individual comparison). Comparison of mean radii (**e**) and mean distances (**f**) for 6 male lobes and 6 female lobes. On each plot the two regression lines x/y and y/x are shown. For radii**,** in **a, c** and **e,** the sex dimorphic glomeruli (43, 44 and 45) were not included in the determination of the regression lines.

Fig. 6 Maximum projections of stacks of confocal sections of the deuto- and tritocerebrum and the suboesophageal ganglion of *Cydia molesta* showing backfills from male (**a**, z from 7 to 47 µm) and female (**b**, z from 21 to 38 µm) palp- and male (**c**, z from 6 to 54 µm) and female (**d**, z from 9 to 58 µm) antennal backfills. d, dorsal; v, ventral; 45, ordinary glomerulus receiving input from the palps. Scale bar = 50 µm.

Table 1. Differences in the presence of glomeruli between antennal lobes in the three males (*M*1, *M*2, *M*3) and females (*F*1, *F*2, *F*3) of *Cydia molesta*. Glomeruli are given the same name as in the antennal lobe sections (Figs. 1 and 2) and reconstruction (Fig. 3).

	Males						Females						
	*M*1		*M*2		*M*3		*F*1		*F*2		*F*3		Colour code in Fig. 3
	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	Left	Right	
Total number	50	50	50	50	49	49	51	51	50	51	52	53	–
Sexually isomorphic													
Normal	1 to 35						1 to 35						
Anomalous missing	36	36	36	36	–	36	36	36	36	36	36	36	Yellow
	37	37	37	37	37	37	37	37	37	37	–	–	
	38	38	38	38	38	38	–	–	–	–	38	38	
	39	39	39	39	39	39	–	–	–	–	39	39	
	40	40	40	40	40	–	40	40	40	40	40	40	
	41	41	41	41	41	41	41	41	–	–	41	41	
	42	42	–	–	42	42	42	42	42	42	42	42	
Sexually dimorphic													
	43	43	43	43	43	43	43	43	43	43	43	43	Green
	44	44	44	44	44	44	44	44	44	44	44	44	
	45	45	45	45	45	45	45	45	45	45	45	45	
Sexually specific													
Male	a	a	a	a	a	a	–	–	–	–	–	–	Red
	b	b	b	b	b	b	–	–	–	–	–	–	
	c	c	c	c	c	c	–	–	–	–	–	–	
	d	d	d	d	d	d	–	–	–	–	–	–	
	e	e	e	e	e	e	–	–	–	–	–	–	
Female	–	–	–	–	–	–	f	f	f	f	f	f	Blue
	–	–	–	–	–	–	g	g	g	g	g	g	
	–	–	–	–	–	–	h	h	h	h	h	h	
	–	–	–	–	–	–	i	i	i	i	i	i	
	–	–	–	–	–	–	j	j	j	j	j	j	
	–	–	–	–	–	–	k	k	k	k	k	k	
Female anomalous missing	–	–	–	–	–	–	l	l	–	–	l	l	Yellow
	–	–	–	–	–	–	–	–	m	m	m	m	
	–	–	–	–	–	–	n	n	n	n	–	–	
Anomalous supernumerary	–	–	–	–	–	–	–	–	–	o	–	–	not shown
	–	–	–	–	–	–	–	–	–	–	–	p	
	–	–	q	q	–	–	–	–	–	–	–	–	

Table 2. Coefficients of correlation (*r*) of size and location of homologous glomeruli in male (*M*1, *M*2, *M*3) and female (*F*1, *F*2, *F*3) antennal lobes of *Cydia molesta*.

Comparison		n° glomeruli paired	Size r [a]	Location r [b]
Intra-individual[c]	*M*1	50	0,9078	0,9836
	*M*2	50	0,7118	0,8754
	*M*3	49	0,8483	0,9799
	*F*1	51	0,8480	0,9677
	*F*2	50	0,7487	0,9504
	*F*3	52	0,6644	0,9151
Inter-individual[d]	*M*1/*M*2	49	0,8291	0,8039
	*M*2/*M*3	49	0,7912	0,7645
	*M*1/*M*3	50	0,7095	0,6574
	*F*1/*F*2	49	0,6542	0,5095
	*F*2/*F*3	48	0,5993	0,4160
	*F*1/*F*3	49	0,7501	0,7197
Inter-sexual	*M*/*F*	45[e]	0.4648	0.6118

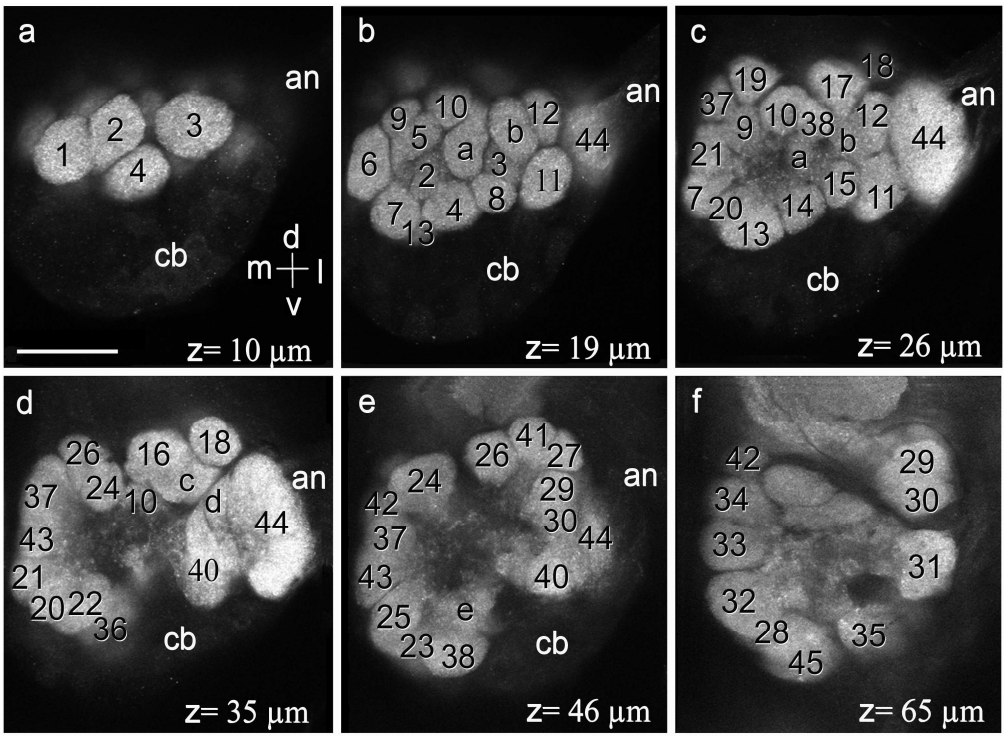
[a] Size of glomeruli measured by their radius. All coefficients of correlation significantly different from 0 with p values < 0.001, except for inter-sexual comparison (p = 0.002).

[b] Location of glomeruli measured by their distance to the centre of the lobe. All coefficients of correlation significantly different from 0 with p values < 0.001.

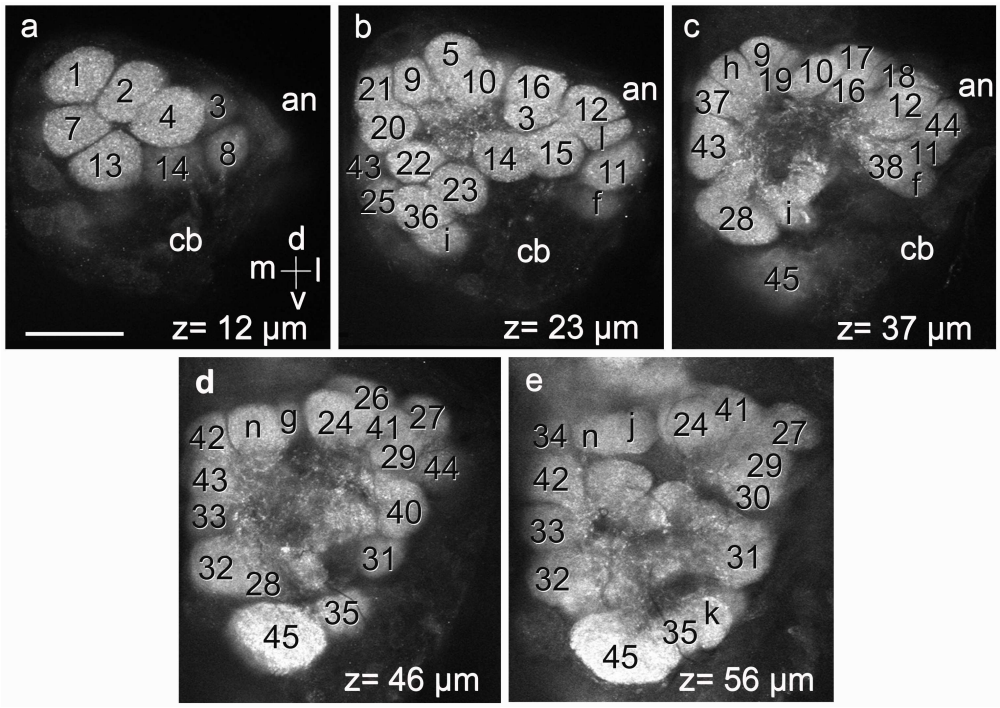
[c] Intra-individual: comparison of antennal lobes of each individual, right *vs*. left.

[d] Inter-individual: comparison of average lobes (right-left), male *vs*. male, female *vs*. female.

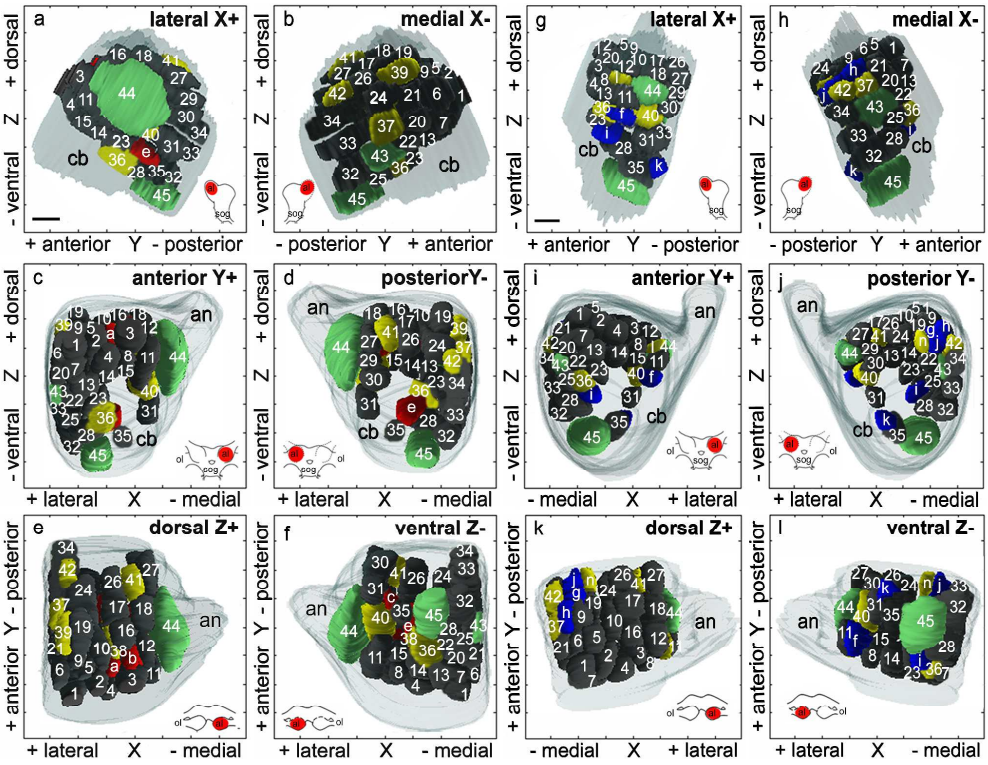
[e] Inter-sexual: comparison of average lobe of males *vs*. average lobe of females on all 45 paired glomeruli for location and on 42 isomorphic pairs for radii (glomeruli 43, 44 and 45 excluded).



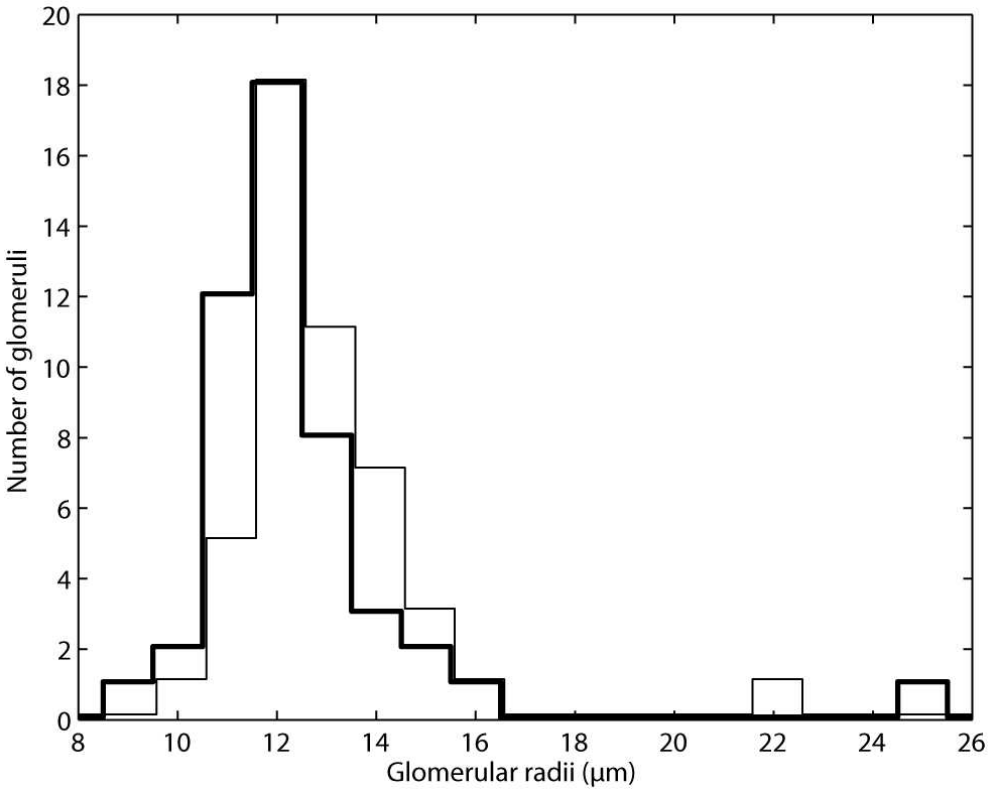
128x94mm (600 x 600 DPI)



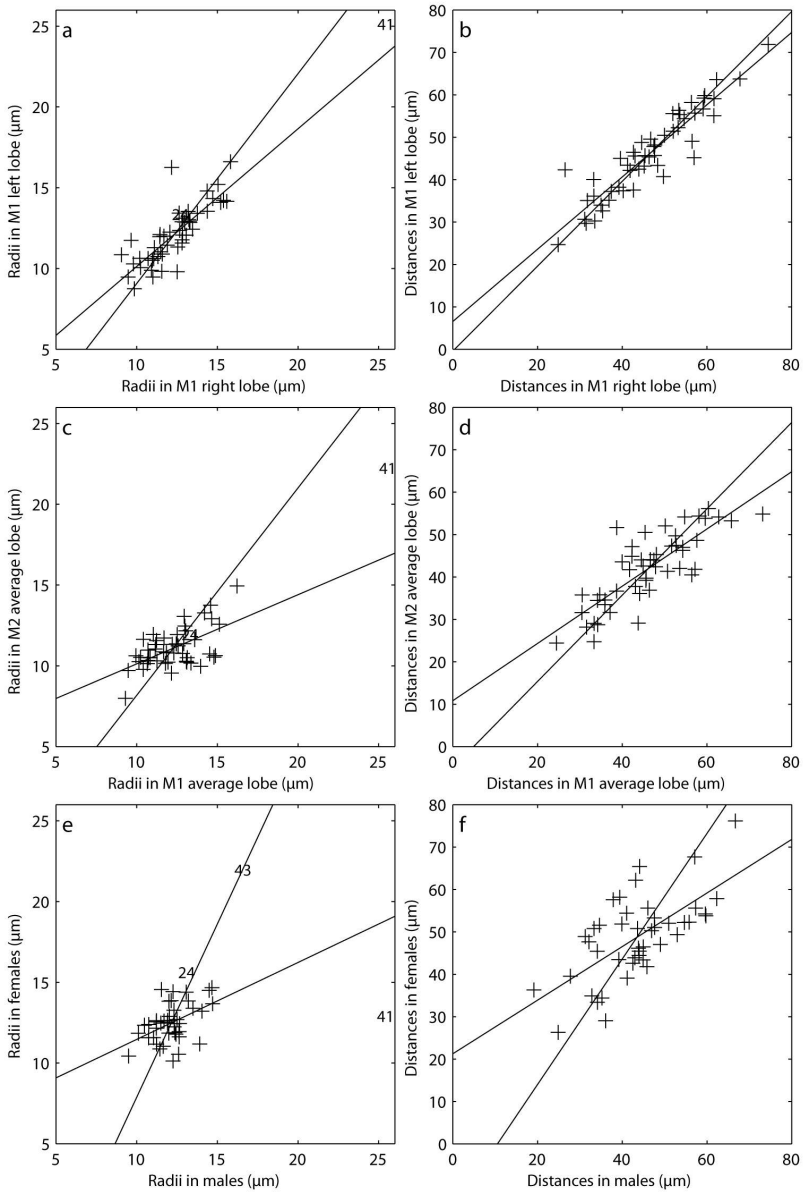
128x90mm (600 x 600 DPI)



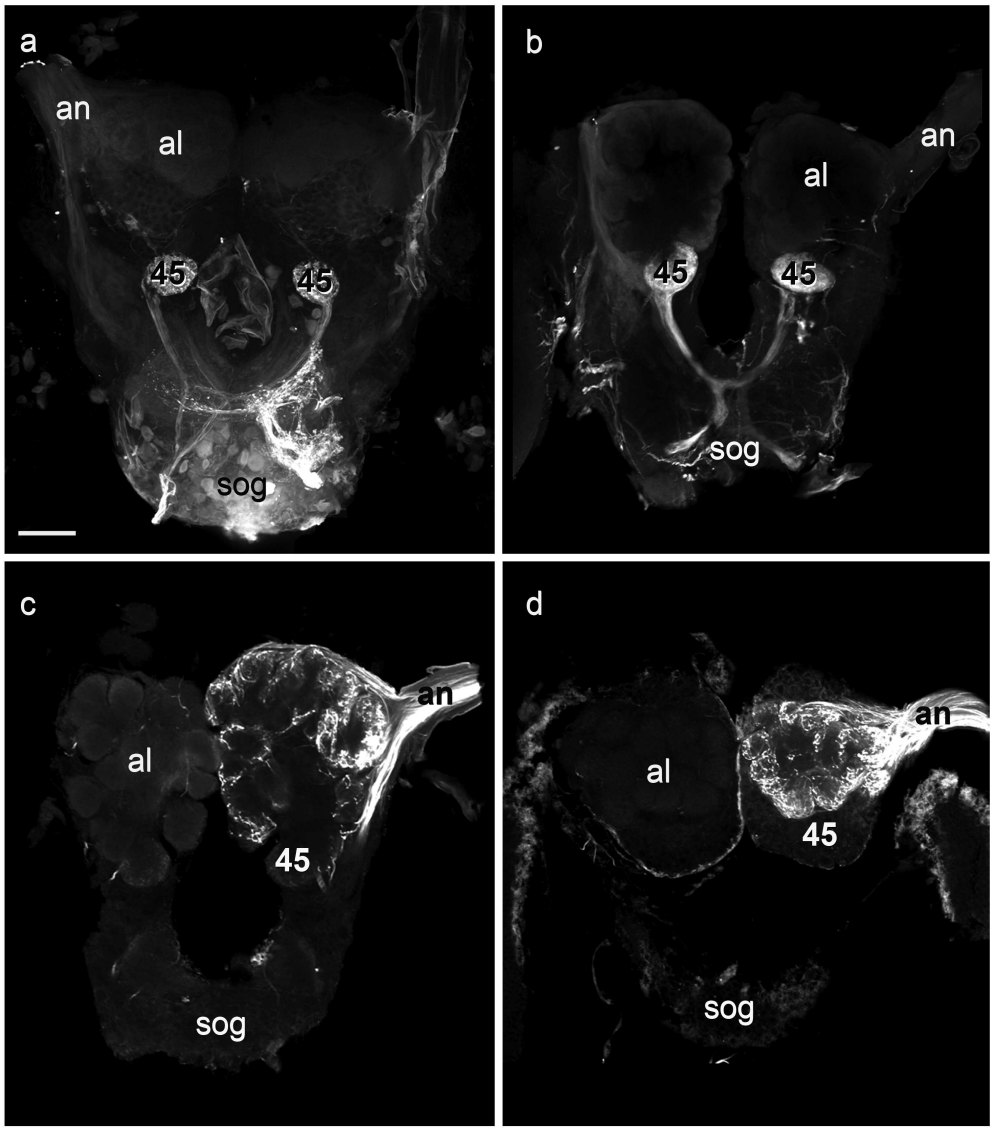
173x132mm (600 x 600 DPI)



84x67mm (300 x 300 DPI)



129x191mm (300 x 300 DPI)



128x146mm (600 x 600 DPI)